BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

141616541

IN THE UNITED STATES PATENT AND TRADE MARK OFFICE

DECLARATION UNDER 35 U.S.C §1.132

Sir:

1. I, Peter Laurence Molloy am a co-inventor of United States of America Patent Application No 09/914651, entitled "Regulatory constructs comprising intron 3 of prostate specific membrane antigen gene" (the Application) and am accordingly familiar with the disclosure of this Application.

2. I have been involved in the design and execution of further experiments relating to preclinical evaluation of a prostate targeted gene directed enzyme prodrug therapy delivered by ovine atadenovirus. These experiments involved the use of the enhancer element disclosed and claimed in the Application.

3. The experiments were carried out to test the efficacy of atadenovirus-delivered prostate-targeted gene-directed enzyme prodrug therapy (GDEPT) against human prostate cancer xenografts. Prostatecancer xenografts in nude mice were virally transduced with ovine atadenovirus (OadV63) carrying the PNP (purine nucleoside phophorylase) gene controlled by PSMEPb (prostate-directed promoter and enhancer). The results indicate that PNP-GDEPT delivered by OadV623 reduced human prostate cancer xenograft growth in mice. These further experiments and results are described in detail below.

4. MATERIALS AND METHODS

The cell lines used for xenografts in nude mice included LNCaP-LN3 (LN3) prostate cancer cells that are androgen-sensitive (1) from Dr CA Pettaway (MD Anderson Cancer Centre, Houston, TX, USA). PC3 androgen-refractory prostate cancer cells were from ATCC (Rockville, MD). Media and chemicals were from Invitrogen, Life Technologies, Grand Island, NY unless otherwise stated. LN3 cells were grown in a 1:1 mix of RPMI 1640 and F12-K, and PC3 cells in RPMI 1640 medium supplemented with 2 mM L-glutamine, 0.2M HEPES buffer, 50U/ml penicillin G, 50μg/ml streptomycin sulfate, 0.85g/L $NaHCO_3$ (Sigma Chemical Co, St. Louis, MO) and 10% fetal bovine serum. Mycoplasma-free cells in

log phase were harvested with 0.05% trypsin/0.5 mM EDTA. 10X cells were implanted subcutaneously in nude mice as described (8).

The nude mice used in the experiments were male BALB/c athymic nude mice aged 10-17 weeks. The mice were bred at the Biological Resources Centre, University of New South Wales, Sydney, Australia and maintained under SPF conditions on mouse chow and acidified water. Experiments were approved by the University's Animal Care and Ethics Committee.

Recombinant atadenovirus production included the use of two viruses both containing the PNP gene, that differed only in their promoters. OAdV220 uses the RSV promoter, such that PNP will be constitutively expressed (3), whereas OAdV623 contains prostate-directed promoter elements. Gene expression cassettes were constructed in plasmids by linking the PSMEPb or RSV promoter to sequences for the *E coli* DeoD gene that encodes PNP (Genbank Accession Number M60917) and the polyadenylation signal from bovine growth hormone. Recombinant OAdV623 and OAdV220 were constructed by cloning the PSMEPb or RSV-controlled expression cassettes into ApaI/NotI sites in pOAdV600 or pOAdV200, respectively, plasmids that contain an infectious OAdV genome (Figure 1) (4, 5). These viruses were used in Example 12 of the patent where specificity of expression following infection by OAdV623 of prostate and other cell lines *in vitro* was demonstrated.

As can be seen in Figure 1 OAdV623 includes the enhancer element of the Application. Viruses were rescued and propagated in CSL503 cells (sheep fetal lung cells), purified on CsCl density gradients (6), desalted using NAP-25 columns (Amersham Pharmacia Biotech AB, Uppsala), and stored in buffer [10mM Tris HCl, pH8, sucrose 8.5% (w/v) and 0.5% polyethylene glycol 400 (v/v)]. To improve infectivity for *in vivo* studies, viruses were formulated prior to injection in buffer containing a cationic lipid, 10□μM CS087 (T-$K_3C_7TL_3$) (7), and this was used as the vehicle control.

To assess PNP activity following *in vivo* treatment, mice were sacrificed at selected times after intratumoral injection of OAdV623 at several doses. Tumors were homogenized, and PNP activity determined as above (8, 3). Briefly, 500 μg protein was incubated with 500 pmoles of 6MPDR (a substrate for PNP) for 2 hours at 37°C before being analysed by reverse phase HPLC for conversion to 6MP.

Human prostate carcinoma cells were grown in adult male nude mice by subcutaneous sc) injection of 2×10^6 LN3 or 2.5×10^6 PC3 cells in the rear flank. Once the tumors reached ~5×5mm, 20µl of OAdV623 or vehicle was injected into the tumors using 0.25 ml disposable syringes with a 29-gauge needle that was moved to various sites in the tumor to maximize viral distribution. The prodrug, fludarabine phosphate (Schering, AG Germany), was given intraperitoneally (ip) daily for the following 5 days at 75mg/m^2/day. Mice were grouped as: (1) intratumoral injection with vehicle and ip saline (vehicle control); (2) intratumoral vehicle and ip fludarabine (fludarabine control); (3) intratumoral 10^{10} VP OAdV623 and ip fludarabine (GDEPT 10^{10} VP), and (4) intratumoral 3.19×10^{10} VP OAdV623 and ip fludarabine. (GDEPT 3×10^{10} VP, for the PC3 study only). Mice were sacrificed when the tumor was 15×15mm or if body weight loss exceeded 20%. At sacrifice, a portion of the tumor and several organs (draining lymph nodes, liver, spleen, kidney, lung, gut) from 3 mice/group were fixed for histology. In a separate experiment, PC3 tumors given GDEPT at 10^{10} VP were harvested 3 and 7 days post-virus injection for histology and immunohistochemistry.

Tumor growth was evaluated by measuring tumors thrice weekly and tumor volumes were calculated as described (9). Tumor growth was normalized for differing starting sizes by calculating Relative Tumor Volume (RTV), the ratio of each tumor volume on each day relative to its volume on Day 1. Growth Delay Index was used to evaluate the change in tumor growth due to treatment. The number of days for the median RTV of each group to reach 2 (i.e. when the tumors had doubled in size) was measured, and the Growth Delay Index was this value for the treated groups divided by the vehicle control. A second parameter used to compare tumor size among groups was % Tumor Growth Inhibition, which can be evaluated on any measurement day before the first animal death due to tumor size in any group. This was calculated on selected days as 100 – (median RTV treated divided by median RTV vehicle control × 100).

The extent of tumor necrosis or leucocyte infiltration into the tumor, the development of spontaneous metastases and toxic damage in various tissues were assessed. Tissues were fixed in FAA buffer (50% ethanol, 3.2% formaldehyde, 4% glacial acetic acid) and paraffin embedded. Tissue sections (5µm) were stained with hematoxylin and eosin (H&E).

Apoptosis was detected using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) technique (10) with an in situ death detection kit, AP (Roche Diagnostics, Mannheim, Germany) as per the manufacturer's instructions. Stained

sections were examined using a microscope (Leitz Laborlux S; Leica, Wetzlar, Germany) coupled to a video camera (Sony Hyper HAD, color CCD-IRIS/RGB, model DXC-151AP), connected to a computer equipped with an image analysis software program (Leica Qwin). From each section, ten high power microscopic fields (HPF) of highest positive areas were randomly selected, counted for positive cells within a fixed frame of 450x450 pixels (0.1520 mm^2), and averaged (number of positive cells/ HPF).

The experiments involved statistical analysis where the data obtained were analyzed using Sigma Stat. To test for differences in tumor growth, RTV was compared between groups by one-way analysis of variance (ANOVA on ranks), and where significant, a Dunn's post test was performed to find the groups that differed. Image analysis results were tested by the unpaired t test. For mouse survival, treated and control groups were compared by a Logrank Test. A value of $P < 0.05$ was considered significant.

5. RESULTS

To confirm gene transfer by different vector doses and to examine the kinetics of gene expression *in vivo*, PNP expression in tumor homogenates was assessed after intratumoral injection of OAdV623. A single injection of 10^{10}, 10^9 or 10^8 VP into LN3 tumors generated an apparent dose-response of PNP activity (Figure 2A), with a statistically significant difference between 10^8 VP and 10^{10} VP (137 ± 31, n=10 v 38 ± 11, n=8, nmol 6MPDR converted to 6MP/mg protein/h (PNP conversion units), mean ± SEM, $P<0.01$). Injection of PC3 tumors with 10^{10} VP produced 165 ± 32 (n=8), 205 ± 30 (n=8), and 274 ± 25 (n=7) PNP conversion units on days 1, 3 and 6 respectively (Figure 2B). This result confirmed that OAdV623 effectively delivered the PNP gene *in vivo* and that PNP activity persisted for at least 6 days.

Previous data from work in nude and immunocompetent mice showed that injection of the wildtype ovine atadaenovirus (8) or the homologue, OAdV220 (11) alone did not affect tumor growth. Fludarabine alone also did not significantly affect LN3 tumor growth compared to vehicle control (Figure 3A). Tumour volume in the GDEPT group was suppressed from day 7 onwards, and in the resulting growth inhibition analysis, this reached statistical significance on Day 22 ($P<0.05$, Table 1), with median RTV less than half of vehicle control from Day 20. Tumors in the GDEPT group took more than twice as long as the vehicle control to double in size, with a tumor growth delay index of 2.3 (Table 2).

Three GDEPT treated mice were cured of their tumors by day 17; control groups showed no cures.

GDEPT efficacy on PC3 tumor growth was also assessed. At both 10^{10} VP and $3.19x10^{10}$ VP, GDEPT significantly suppressed PC3 tumor growth for at least 15 days, following which tumor growth rate was similar in all groups (Figure 3B). Analysis of tumor growth inhibition showed that tumors treated with 10^{10} VP were 41-46% smaller compared to vehicle control, for at least 14 days of the treatment period (days 13-27) ($P<0.001$, Table 1). Even greater reduction in tumor size was observed in tumors administered with the higher dose, showing a dose-response to the treatment, although this was not statistically significant. Compared to the vehicle control, tumors in both of the GDEPT groups took more than twice as long to double in size (Table 2). Two tumors in the GDEPT group were cured after days 18 and 54, respectively.

The effect of GDEPT on survival of tumor-bearing nude mice was also assessed. All LN3-bearing mice in the vehicle and fludarabine control groups were dead by day 45 (Figure 4A); those treated with GDEPT showed 67% survival on day 45. The median survival time for GDEPT treated mice was 48 days, compared with 27 days for vehicle and 36 days for fludarabine alone ($P=0.02$). The median survival time for PC3-bearing mice (Figure 4B) treated with GDEPT at both virus doses was >80 days (undefined when the experiment ended), compared with 59 and 54 days in vehicle-treated and fludarabine control groups. At day 59, survival was 67% and 73% in mice given GDEPT with 10^{10} VP or $3x10^{10}$ VP, respectively.

H&E stained tumor sections from 3 mice per group, evaluated at different times after vector treatment, showed different responses in LN3 and PC3 tumors. On day 3, LN3 tumors from mice given GDEPT showed less necrosis (34 ± 18%) than those from vehicle and fludarabine controls (76 ± 18% and 73 ± 13% necrosis respectively). In contrast, GDEPT treated PC3 tumors showed significantly higher levels of necrosis on day 3 (55 ± 16%, 57 ± 2% for the 1 or $3x10^{10}$ VP, respectively) when compared with vehicle (7 ± 3%) and fludarabine (10 ± 5%) controls, but by day 7 and later, varying degrees of necrosis were seen. Minor to moderate immune cell infiltration was seen both at the periphery and in the center of both LN3 on day 3 and PC3 tumors examined on days 3 and 7, but there was no difference between experimental groups. No signs of toxic damage from treatment were seen on histological assessment of other tissues obtained at sacrifice during the treatment experiments, including liver, spleen, kidney, lung and gut. PC3 tumor cell

growth occurred in 1 or 2 of draining lymph nodes from 3 mice in both GDEPT and control groups.

To investigate how GDEPT mediates cell death *in vivo*, the TUNEL assay was performed on PC3 tumors harvested 3 and 7 days after treatment. Scattered apoptotic cells were seen throughout the tumors of vehicle and fludarabine controls; apoptosis was increased in tumors of the GDEPT groups in necrotic areas, associated with polymorphonuclear cell and monocyte infiltration, and in non-necrotic areas (Figure 5). GDEPT led to over a 2 fold increase in the number of apoptotic cells in PC3 tumors compared with controls on day 3 (GDEPT $3x10^{10}$ VP, 58 ± 2; GDEPT 10^{10} VP, 6 ± 5; fludarabine control, 19 ± 2; vehicle control, 19 ± 3; $P<0.01$), and showed a similar trend on day 7 (GDEPT at $1x10^{10}$ or $3x10^{10}$ VP: 52 ± 6, 52 ± 4 respectively; controls: 35 ± 4), but this was not significant.

6. In my opinion the experiments described above and those set out in the Application clearly demonstrate that the enhancer element disclosed in the Application enables expression of a coding sequence within a cell both *in vivo* and *in vitro*.

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements are made with the knowledge that wilful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of the Title 18 of the United States Code, and that such wilful false statements may jeopardise the validity of this application or any patent issuing thereon.

Dated: March 16th 2004

PETER LAURENCE MOLLOY

REFERENCES

1. Pettaway, C. A., Pathak, S., Greene, G., Ramirez, E., Wilson, M. R., Killion, J. J., Fidler, I. J.: Selection of highly metastatic variants of different human prostate carcinomas using orthotopic implantation in nude mice Clin. Cancer Res., 2:1627-1636, 1996.

2. Freytag, S. O., Khil, M., Stricker, H., Peabody, J., Menon, M., Deperalta-Venturina, M., Nafziger, D., Pegg, J., Paielli, D., Brown, S., Barton, K., Lu, M., Aguilar-Cordova, E., Kim, J. H.: Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. Cancer Res., 62: 4968-4976, 2002

3. Voeks, D., Martiniello-Wilks, R., Madden, V., Smith, K., Bennetts, E., Both, G. W., Russell, P. J.: Gene therapy for prostate cancer delivered by ovine adenovirus and mediated by purine nucleoside phosphorylase and fludarabine in mouse models. Gene Ther., 9: 759-768, 2002.

4. Vrati, S., Macavoy, E. S., Xu, Z. Z., Smole, C., Boyle, D. B., Both, G. W.: Construction and transfection of ovine adenovirus genomic clones to rescue modified viruses. Virology, 220:200-203, 1996.

5. Xu, Z. Z., Hyatt, A., Boyle, D. B., Both, G. W.: Construction of ovine adenovirus recombinants by gene insertion or deletion of related terminal region sequences. Virology, 230: 62-71, 1997.

6. Boyle, D. B., Pye, A. D., Kockerhans, R., Adair, B. M., Vrati, S., Both, G. W.: Characterisation of Australian ovine adenovirus isolates. Vet. Microbiol., 41: 281-291, 1994.

7. Cameron, F. H., Moghaddam, M. J., Bender, V. J., Whittaker, R. G., Mott, M., Lockett, T. J.: A transfection compound series based on a versatile Tris linkage. Biochim. Biophys. Acta, 1417:37-50, 1999.

8. Martiniello, R., Garcia-Aragon, J., Daja, M., Russell, P., Both, G. W., Molloy, P. L., Lockett, L. J., Russell, P. J.: *In vivo* gene therapy for prostate cancer: preclinical evaluation of two different enzyme-prodrug systems delivered by identical adenovirus vectors. Hum. Gene Ther., 9:1617-1626, 1998.

9. Russell, P. J., Raghavan, D., Gregory, P., Philips, J., Wills, E. J., Jelbart, M., Wass, J., Zbroja, R. A., Vincent, P. C.: Bladder cancer xenografts: a model of tumor cell heterogeneity. Cancer Res., 46:2035-2040, 1986.

10. Shirakawa, T., Gotoh, A., Wada, Y., Kamidono, S., Ko, S. C., Kao, C., Gardner, T. A., Chung, L. W.: Tissue-specific promoters in gene therapy for the treatment of prostate cancer. Mol. Urol., 4:73-82, 2000.

11. Eastham, J. A., Chen, S.-H., Sehgal, I., Yang, G., Timme, T. L., Hall, S. J., Woo, S. L., Thompson, T. C.: Prostate cancer gene therapy.: Herpes simplex virus thymidine kinase gene transduction followed by ganciclovir in mouse and human prostate cancer models. Hum. Gene Ther., 7:515-523, 1996.

Table 1 Effect of GDEPT on LN3 (top panels) and PC3 (bottom panels) tumor growth.

Treatment group LN3	N	Median relative tumor volume			% Tumor growth inhibition		
		Day 17	Day 20	Day 22	Day 17	Day 20	Day 22
Control	9	3.4	4.1	4.9	0	0	0
Fludarabine	12	4.3	4.9	6.8	-29	-20	-40
GDEPT 10^{10}VP	12	1.1	2.1	2.0 *	41	50	59
Treatment group PC3 **		Day 13	Day 20	Day 27	Day 13	Day 20	Day 27
Control	11	2.0	3.1	4.2	0	0	0
Fludarabine	12	2.2	3.1	4.4	-11	1	-5
GDEPT 10^{10} VP	15	1.2	1.7	2.3	41	46	44
GDEPT 3.19×10^{10} VP	15	1.0	1.3	2.1	53	57	50

* $P<0.05$ versus control (ANOVA on ranks; Dunn's test)

** $P<0.001$ for all PC3 GDEPT groups versus control (multiple comparison Student-Newman-Keuls Method)

The Relative Tumour Volume (RTV) was calculated as the ratio of the tumour volume on Day (x) to its volume on Day (initial). % Tumor Growth Inhibition [100 - (median RTV treated / median RTV control x 100] was evaluated on several different days before the first animal death due to tumor size in any group, and representative values are shown here. A value of 50 indicates that the treated group was 50% smaller than the vehicle control. GDEPT treatment consisted of intratumoral OAdV623 at the indicated doses (VP = virus particles) on day 0, followed by fludarabine phosphate for 5 days.

TABLE 2. Tumor Growth Delay analysis for LN3 and PC3 mouse xenograft studies.

Group	Time to reach 200% (Days)		Tumor Growth Delay Index	
	LN3	PC3	LN3	PC3
Vehicle control	7	11.7	1	1
Fludarabine control	5	9.0	0.7	0.8
GDEPT 10^{10}VP	16	23.2	2.3	2.0
GDEPT $3.19x10^{10}$ VP	-	25.2	-	2.2

The time for tumours in each treatment group to reach 200% of initial size (RTV = 2) was measured from the graph of median RTV vs Days post treatment. Growth Delay Index = Median time for treated groups (days) / Median time for vehicle control (days), where a value of 2 indicates the treated group took twice as long as the control to double in size. GDEPT treatment consisted of intratumoral OAdV623 at the indicated doses (VP = virus particles) on day 0, followed by fludarabine phosphate for 5 days. N=9 to 15, refer to Fig 3.

FIGURE LEGENDS

Figure 1. Structure of OAdV220 and OAdV623. The position of expression cassette in Sites I or III are shown. The expression cassette comprises the PSME, probasin (Pb430) or RSV 3' long terminal repeat (LTR) promoters, the PNP gene and 3' polyadenylation sequences from the bovine growth hormone gene (BGH), flanked by ApaI and NotI restriction sites.

Figure 2. PNP expression from OAdV623 delivered *in vivo*. PNP expression by tumor homogenates was evaluated by the conversion of 6-methyl-9-(2-deoxy-β-D-erythro-pentofuranosyl) purine (6MPDR) to 6-methylpurine (6MP) and detected using HPLC (4, 16). The conversion rate for individual tumors is presented as PNP Conversion Units, nanomoles 6MPDR converted to 6MP/mg tumor protein/hour (nmol 6MPDR/mg protein/h). A: LN3 tumors were harvested 2 days after intratumoral injection with 10^{10} VP (n=10), 10^{9} VP (n=10), 10^{8} VP (n=14) or nil (n=8) OAdV623. (B) PC3 tumors were harvested 1 (n=8), 3 (n=8) or 6 (n=7) days after intratumoral injection with 10^{10} VP OAdV623. Data from individual mice are presented, "—" indicates mean.

Figure 3. Semi-log plots of effect of OAdV623-GDEPT on tumor growth in nude mice. (A) LN3-tumor bearing mice were treated with: intratumoral vehicle and saline ip daily for 5 days thereafter (vehicle); intratumoral vehicle and fludarabine phosphate ip (fludarabine); intratumoral OAdV623 (10^{10} VP) and fludarabine ip (GDEPT). Data are presented as median relative tumor volume (RTV) vs time (days post intratumoral treatment). Inset: mean tumor volume ± SEM. This study has been performed 3 times with similar results. (B) PC3 tumor bearing mice were treated with: vehicle control; fludarabine control; OAdV623 10^{10}VP plus fludarabine; or OAdV623 $3.19x10^{10}$VP plus fludarabine . Inset: mean tumor volume ± SEM.

Figure 4. Survival of prostate tumor bearing nude mice after OAdV623 GDEPT. A, LN3-tumor bearing mice; B, PC3-tumor bearing mice. Mice were sacrificed when tumors reached 15x15mm (1767.1 mm^3), or if they lost > 20% body weight. Data from mice that died of non-tumor related causes were censored. Mice were treated as for Figure 3.

Figure 5. Induction of apoptosis in PC3 tumors by GDEPT treatment. Apoptotic cells in PC3 tumors were detected by in situ TUNEL assay. Representative images of apoptosis in PC3 tumors harvested on day 3: (A) control tumor. Small numbers of apoptotic cells were seen in necrotic and non-necrotic areas in vehicle and fludarabine control tumors. (B) GDEPT tumor, necrotic area. Apoptosis associated with neutrophil and monocyte infiltration. Positive cells are stained red. Microscope magnification 40x.